Potential role of glutamate neurotransmission in the pathogenesis of ischemic brain damage and of depression. Effects of L-kynurenine on the survival of the hippocampal neurons and on the corticocerebral blood flow in ischemic animal models

Ph.D. thesis

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SCIENTIFIC PUBLICATIONS OF THE AUTHOR OF THE PH.D. THESIS

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LIST OF ABBREVIATIONS

AMPA: α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid,

BBB: blood-brain barrier

BDNF: brain-derived nerve growth factor,

cCBF: cortical cerebral blood flow,

EAA: extracellular amino acid,

fEPSPs: field excitatory postsynaptic potentials,

FJ-B: Fluoro Jade B,

FJ+: Fluoro Jade positive

GABA: gamma-aminobutyric acid

Glu: glutamate,

Gly: glycine,

HFS: high-frequency stimulation,

HR: heart rate,

IDO: indolamine 2,3-dioxygenase,

IO curves: input-output curves,

KYN: kynurenine,

KYNA: kynurenic acid,

MABP: mean arterial blood pressure,

L-NAME: nitro-L-arginine methyl ester,

LTP: long-term potentiation,

NA: noradrenaline,

NeuN: anti-neuronal nuclei,

NMDA: *N*-methyl-D-aspartate,

NO: nitric oxide,

NOS: nitric oxide synthase,

PSD: post-stroke depression,

PROB: probenecid,

QUIN: quinolinic acid,

SC: sham control,

SER: serotonin,

SSRI: selective serotonin reuptake inhibitor,

TRP: tryptophan,

4VO: four-vessel occluded

INTRODUCTION

Kynurenine pathway. Kynurenic acid – a glutamate antagonist

In addition to the process of protein synthesis, tryptophan (TRP) is metabolized along several separate pathways in mammals. The most commonly known is the serotonergic pathway, yielding 5-hydroxytryptophan and then serotonin (SER). The less well-known, but actually the major alternative route for the TRP metabolism, is through the kynurenine (KYN) pathway. A central material of the pathway is KYN, which can be metabolized in two separate ways: one branch results in the formation of kynurenic acid (KYNA), while the other leads to quinolinic acid (QUIN), the precursor of nicotinamide adenine dinucleotide (Fig. 1).

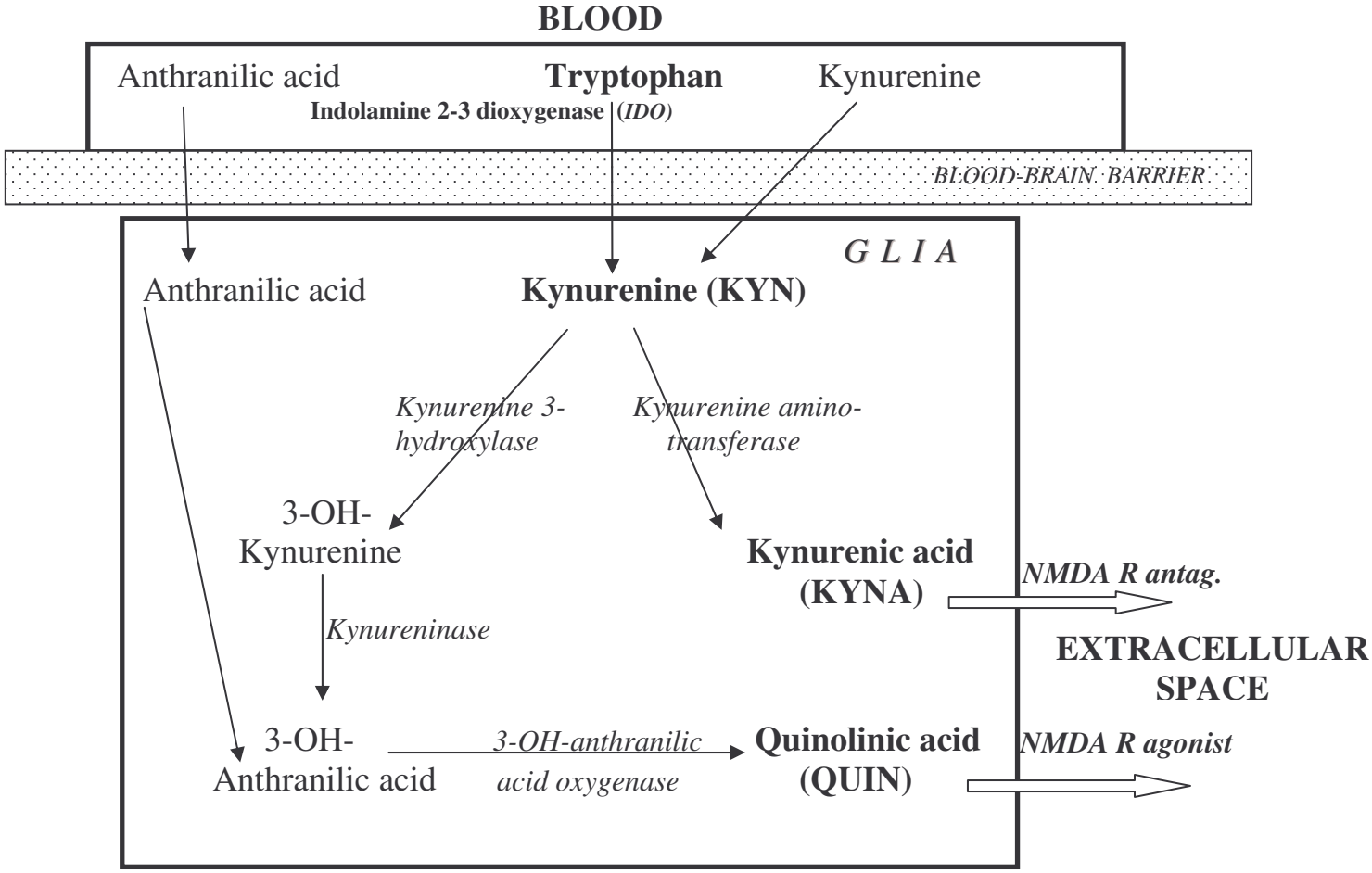

FIGURE 1. Metabolism of KYN in the glial cell.

Importance has been attached to KYNA by virtue of the fact that this is the only known endogenous excitatory amino acid (EAA) receptor blockers with a broad spectrum of antagonistic properties in supraphysiological concentrations[53,74]. KYNA exhibits a

particularly high affinity for the glycine (Gly)-binding site of the *N*-methyl-D-aspartate (NMDA) receptor, blocking its activity in low micromolar concentrations[26]. A blockade of the glutamate (Glu)-binding site of the NMDA receptor complex requires concentrations 10-20-times higher than those for the Gly site, whereas KYNA exhibits a weak antagonistic effect on the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and kainate receptors[5]. A recently identified site of action for KYNA is the α7-nicotinic acetylcholine receptor, which it blocks in a noncompetitive manner[22]. Interestingly, a previously unrecognized higher-affinity positive modulatory binding site at the AMPA receptor has been discovered[54].

On the other hand, QUIN is a specific agonist at the NMDA receptors, and a potent neurotoxin with an additional marked free radical-producing property.

KYN metabolites are also present in the serum (in higher concentrations), although its precise physiological function in the peripheral organs is not yet clear.

The cellular uptake of KYN occurs rapidly, predominantly in the astrocytes and microglial cells. While KYNA production is regulated by sophisticated mechanisms, no re-uptake or catabolic process for the removal of extracellular KYNA has so far been identified. Probenecid (PROB) is a substance which is known to inhibit KYNA excretion from the extracellular fluid.

Summary: QUIN may result in excitotoxic neuronal cell death, while in stark contrast, the other KYN pathway metabolite, KYNA, protects neurons. There are some diseases where elevations of QUIN in the brain have been implicated, *e.g.* CNS inflammation, stroke, traumatic brain injury, and certain neurodegenerative disorders. In these states, hyperactivity of Glu receptors in certain areas of the brain occurs, causing excitotoxic cell death. On the other hand, in diseases with cognitive alterations, such as Alzheimer's type dementia and schizophrenia, elevated levels of brain KYNA have been demonstrated which could contribute to cognitive defects by influencing NMDA receptors (Sas and Vécsei, 2003) (rewieved by Robotka et al.[58]). There is another aspect of the importance of the neuroactive kynurenines: in certain pathological states, the pathogenesis of which involves (among others) an excitotoxic mechanism, pharmacological elevation of the levels of the Glu antagonist KYNA in the brain can hold promise for the amelioration of some devastating consequences of the process if administered fairly early in the disease. Research is continuing to find KYNA

derivatives with better bioavailability, or enzyme inhibitors which can shift the KYN metabolism toward the KYNA branch, thereby elevating KYNA levels and diminishing QUIN concentrations at the same time.

Rationale of L-KYN administration – diminishing glutamate hyperactivity

It is widely accepted that activation of the EAA receptors plays an important role in neuronal death in stroke[11]. It has recently been reported that Glu-induced excitotoxicity and a cellular calcium overload are among the key factors in brain ischemia (14 and reviewed by Sas et al., 1998). By definition, excitotoxicity is a result of overexcitation of the Glu receptors, which can occur in both an acute, ”strong form” and a chronic, ”weak excitotoxicity” fashion. Besides cerebral ischemia, NMDA receptor overactivity can be assumed in the pathogenesis of several acute and chronic neurological diseases, including brain trauma, epilepsy, Parkinsons’s, Huntington’s and Alzheimer’s diseases, amyotrophic lateralsclerosis, mitochondria-related diseases, neuropathic pain, prion- and AIDS diseases, malignant gliomas and perhaps multiple sclerosis. In turn, neuroprotective strategies have utilized antagonists of Glu receptors to prevent excitotoxic neuronal loss, rewieved by Buchan[8]. Being an endogenous NMDA receptor antagonist, KYNA can be one candidate. The topic has been reviewed in detail by Sas et al. (2007b). Use of a naturally occurring substance may diminish the side-effects that often prevent the application of new chemical entities.

However, the use of KYNA itself as a neuroprotective agent can be difficult because it hardly crosses the blood-brain barrier (BBB), whereas L-KYN is easily transported across it by a neutral amino acid carrier[16]. Accordingly, the systemic administration of L- KYN dose-dependently elevates the level of KYNA in the brain[81].

Experimental data and theoretical considerations suggest that KYNA or its metabolic precursor KYN may provide neuroprotection and can thus have therapeutic consequences in neurological diseases[82,64]. We demonstrated earlier that KYN administration decreases the ischemia-induced deterioration of spontaneous alternations (a measure of spatial memory) in gerbils[18].

Depression – hyperactivity of the glutamate receptors in the hippocampus

The number of patients suffering from vascular depression is rapidly growing. The nature of the mechanism linking vascular pathology and depression remains debated.

"Vascular depression" is related to various types of cerebrovascular pathology[1]. Subgroups of it consist of post-stroke depression (PSD) and subcortical ischemic depression[73]. "Vascular depression" is a form of depression that occurs with diffuse or multifocal cerebrovascular lesions, changes which may be related to atherosclerosis, hypertension or myocardial infarction, and of which the patient can be unaware[1]. The lesions are believed to interrupt cortico-subcortical (cortico-limbic and cortico-striatal) pathways which can cause biochemical changes (rather than the localized structural damage itself), leading to alterations in mood.

Vascular depression and "idiosyncratic" depression can share some common features in their pathomechanism. The initial step in the pathomechanism of "idiosyncratic" depression can be chronic stress, followed by hyperactivity of the glucocorticoid axis[67,50]. The Glu-ergic innervation of the hippocampus from the prefrontal cortex is likewise enhanced[56] (Figure 2).

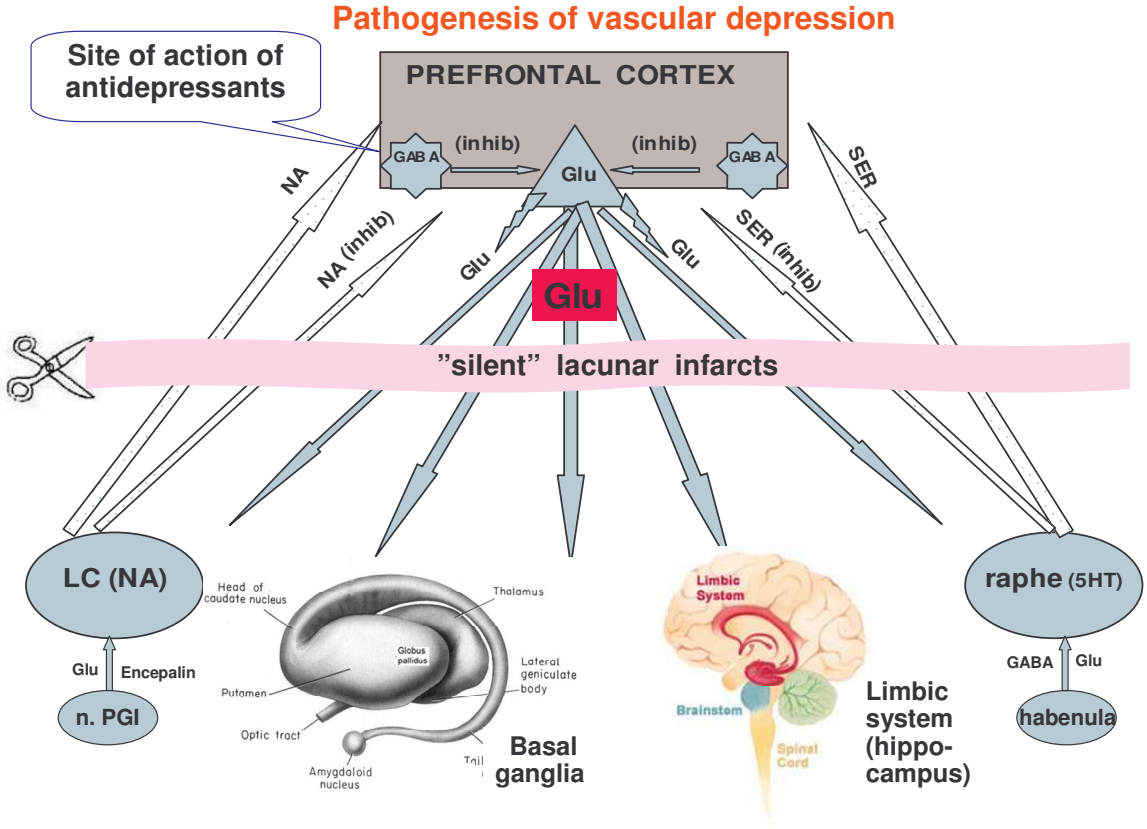

FIGURE 2. Potential pathogenesis of vascular depression. Interaction of the monoamine-Glu system. The prefrontal cortex receives NA-ergic innervation from the locus coeruleus (LC), and SER-ergic innervation from the raphe nucleus (GABA: gamma-aminobutyric acid; NA: noradrenaline; n. PGI: nucl. paragigantocellularis).

In the event of a patient's enhanced vulnerability as concerns depression, when Glu receptor hyperactivity persists for a longer period of time, the condition can cause damage to the hippocampus by several mechanisms: by causing neuronal and glial cell loss[38,65], reducing neurogenesis[15], and diminishing the production of brain-derived nerve growth factor (BDNF)[70], which together can play a role in evoking depressive symptoms reviewed by Sas et al. (2006). Interestingly, cell damage in the hippocampus affects glial cells (in the form of cell loss and dendritic involutions)[60,38] rather than neurons (in contrast with most of the neurodegenerative disorders).

The role of chronic peripheral immune activation results in the subsequent release of proinflammatory cytokines by macrophages and dendritic cells in the brain, and hence chronic low-grade cerebral inflammation has come into focus in recent years[71,34]. Cytokines in the brain can further stimulate the hypothalamo-pituitary-adrenal axis, disturb monoaminergic neurotransmission, and induce the enzyme indolamine 2,3-dioxygenase (IDO) (Fig. 3). This enzyme initiates the catabolism of TRP through the KYN pathway. Heightened TRP catabolism in the KYN pathway toward the QUIN arm has been reported in depression[43,44]. An imbalance between the two arms of the pathway can exert an impact on the Glu-ergic neurotransmission in the brain. It has been suggested that impaired neuroprotection may play an important role in the pathophysiology and treatment resistance of depression.

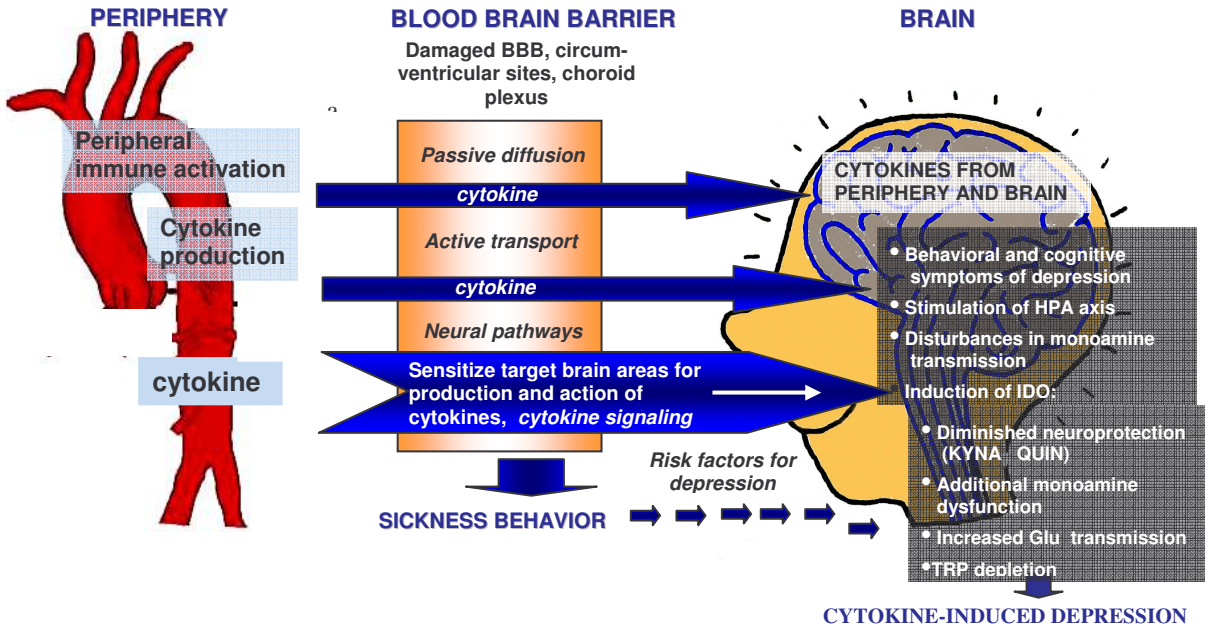

FIGURE 3. Cytokine model of depression. Peripherally released cytokines enter the brain in several ways, where they sensitize target brain areas for further cytokine production.

The immunological hypothesis of PSD has been proposed[72]: the increased production of proinflammatory cytokines resulting from stroke leads to amplification of the inflammatory process and to the activation of IDO and subsequently a widespread decrease in the production of SER; SER-ergic depletion in strategic brain regions such as the paralimbic areas of the left frontal and temporal cortex may ultimately lead to depression[72].

As mentioned above, the prefrontal cortex receives NA-ergic and SER-ergic innervation from the locus coeruleus and raphe nucleus, respectively, and these pathways form synaptic connections with the GABA-ergic interneurons and pyramidal neurons in the prefrontal cortex [52]. These synapses may be the target of antidepressant drugs, where they can elevate synaptic concentrations of NA and SER. The hippocampus is richly innervated by Glu-ergic cortico-limbic pathways. It is supposed that hyperactivity of these pathways damages the hippocampus by several mechanisms. Most antidepressant drugs reverse the impaired neurogenesis and BDNF production in this structure[84] and even possess Glu receptor antagonistic property by binding to the Gly allosteric site or to the ion channel at the NMDA receptor complex[69].

Interruption of the (frontal) cortico-limbic pathways by even subtle vascular lesions in the frontal white matter can lead to biochemical changes that can ultimately cause a special (subcortical-vascular type) depressive syndrome[1].

As concerns the cerebral blood flow (CBF), a considerable number of data support the existence of an impaired regional CBF in major depression, which can be reversed by successful treatment[7] .

OUR OWN STUDIES – OUR AIMS

1. To investigate whether peripheral L-KYN administration has any neuroprotective effect on hippocampal cell survival under global ischemic conditions in the brain.

In our study, we evaluated the effect of L-KYN administration (300 mg/kg i.p.) on the hippocampus of the global ischemic (transiently four-vessel occluded: 4VO) rat brain, administering the L-KYN before and after the ischemic insult. We used the rat 4VO model, mimicking global ischemia, which is a widely recognized model in ischemic studies.

2. How does peripheral L-KYN loading influence the physiological long-term potentiation (LTP) in the ischemic rat hippocampus?

We investigated how KYN (300 mg/kg) treatment influences LTP in the hippocampus in the rat 4VO model. Global ischemia is known to elicit selective, delayed neuronal death. The pyramidal neurons of hippocampal CA1 area are particularly vulnerable. Activity-dependent LTP, a model of synaptic plasticity, also mediated by Glu receptors, may likewise be impaired by global ischemia. This too was addressed in our experiments.

3. Do small doses of peripherally administered L-KYN influence the cortical CBF (cCBF) under normal and chronic ischemic conditions of the brain in the rabbit?

As little amount of information is available on the effects of the KYN system on the CBF, we investigated the effects of low doses (0.3, 1 and 3 mg/kg) of L-KYN on the cCBF under normal and ischemic conditions of the brain in awake rabbits.

4. Do the antidepressant drugs citalopram and fluoxetine have any effects on the cCBF in normal rabbits and in animals with CBF impairement induced by chronic unilateral carotid occlusion?

Antidepressants have proved to be partly effective in vascular depression, but little was eatlier known about their *in vivo* vascular effects. We investigated for the first time the effects of two selective SER reuptake inhibitor antidepressant drugs (SSRIs), citalopram and fluoxetine, on the normal and the carotid ligation-induced ischemic cCBF, and also on the mean arterial blood pressure (MABP) and the heart rate (HR) in awake rabbits.

EXPERIMENT 1

KYNURENINE DIMINISHES THE ISCHEMIA-INDUCED HISTOLOGICAL AND ELECTROPHYSIOLOGICAL DEFICITS IN THE RAT HIPPOCAMPUS

The aim of our study was to reveal whether KYN can rescue the CA1 neurons in the 4VO model in the rat. KYN was administered together with PROB, an organic acid transporter inhibitor, in order to facilitate the brain penetration of KYN (Sas et al., 2008).

Materials and methods

Animals

The study was performed on adult male Wistar rats (n = 43, 280-300 g) maintained under controlled environmental conditions at a temperature of 22±2 °C and a 12-h light/dark cycle. Food and water were available *ad libitum.* The local Animal Ethics Committee had approved all the experiments. The care and use of the experimental animals were in accordance with the 86/609/EEC directive.

Four-vessel occlusion and drug treatment

4VO was carried out as described previously[55]. In brief, the rats were anesthetized with Nembutal (CEVA-PHYLAXIA; 60 mg/kg, i.p.). Both vertebral arteries were occluded by cauterization during careful cooling with iced washing. The wounds were closed, and the animals were allowed to recover for 24 h. On the following day, the animals were subjected to 10-min forebrain ischemia by bilateral occlusion of the carotid arteries with atraumatic clips under ether anesthesia. The body temperature was monitored, and maintained at 37 °C during the surgical procedures, using a thermostatically controlled heating pad. Both vertebral arteries were cauterized, and both common carotid arteries were exposed but not occluded in the sham-operated animals.

The ***rats used for histology*** were divided into 4 groups: sham-operated controls (**SC** group, n = 5), 4VO animals (**4VO** group, n = 7), KYN+PROB-pretreated animals (**KYN+PROB-4VO** group, n = 6) and KYN+PROB-post-treated animals (**4VO-KYN+PROB** group, n = 7).

The ***rats used for electrophysiology*** were divided into 3 groups: sham-operated controls (**SC** group, n = 6), 4VO animals (**4VO** group, n = 6) and KYN+PROB-pretreated animals (**KYN+PROB-4VO** group, n = 6).

KYN (300 mg/kg, i.p.) and PROB (200 mg/kg, i.p.) were administered daily for 5 days: in the ***pretreated group***, then first KYN+PROB administration preceded the 10-min carotid occlusion by 2 h; and the animals were treated at the same time on the next 4 days. In the ***post-treated group,*** the animals received the first KYN+PROB injection at the start of reperfusion. The remaining 4 injections were given at the same time on the next 4 days.

Histological procedures

Ten days after 4VO, the rats were anesthetized with urethane (2.2 g/kg, i.p.), and perfused transcardially with 0.1 M phosphate-buffered saline, and then with 4% paraformaldehyde. The literature reports a considerable range of reperfusion times on transient global ischemia[9,20,46]; our procedure was selected on the basis of these data. The brains were removed from the cranium, post-fixed in formalin, cryopreserved in 20% sucrose (containing 0.05% Na-azide), and sectioned at 32 μm with a cryostat microtome. Serial sections were collected in a cryopreservative solution for storage.

The series of sections were stained for degenerating neurons with Fluoro Jade B® (FJ-B; Chemicon Int.). Six sections were mounted from all animals in each group. A standard protocol was followed: the sections were brush-mounted onto gelatine-subbed slides, and then air-dried for 1-3 days. Before staining, the slides were stored in a thermostat (55 °C) for 30 min. After cooling down to room temperature, the slides were consecutively transferred into 100% ethanol for 3 min, into 70% ethanol for 2 min, into distilled water for 1 min, and into 0.06% potassium permanganate solution for 15 min, and were then rinsed in distilled water.

Staining was performed in 0.004% FJ-B for 30 min in the dark, and after rinsing in distilled water (3 x 1 min) the sections were air-dried in the dark for 1 day.

For anti-neuronal nuclei (NeuN) immunohistochemistry, free-floating sections (32-μm thick) were rinsed in TBS + 0.4% TRITON (TBS: Tris-buffered saline: 0.1 M Tris-HCl [pH 7.4]; 0.15 M NaCl) (3 x 10 min). They were then preincubated with 1% NGS (normal goat serum) + TBS + 0.4% TRITON for 1 h. Primary mouse (NeuN) antibody (Chemicon Int.) at a dilution of 1:2000 in 1% NGS, 0.4% TRITON, 0.05% Na-azide and TBS was then applied overnight at room temperature. After washing in TBS + 0.4% TRITON (3x10 min), sections were incubated with secondary Cytm3-conjugated donkey anti-mouse IgG (H+L) antibody (1:1000; Jackson Immunoresearch, West Grove, PA, USA) for 3 h at room temperature. After

further washing in TBS + 0.4% TRITON (3x10 min), sections were brush-mounted onto gelatine-subbed slides and, then dried in the air for 1 day in the dark.

The locations of FJ-positive (FJ_+) cells were observed with a fluorescence microscope (Olympus BX-51, Tokyo, Japan) with an excitation wavelength of 470-490 nm and an emission wavelength of 520 nm. The NeuN labeling was observed under a fluorescence microscope at an excitation wavelength of 530-550 nm, and an emission wavelength of 590 nm. Fluorescence photomicrographs were obtained with an Olympus DP 70 (Tokyo, Japan) digital imaging system.

The global ischemia-induced damage was examined in both hemispheres, but in each case the hemisphere with the greater extent of injury was evaluated. A section was viewed at 4x magnification and the most dorsal part of area CA1 was chosen. Then, at 20x magnification, the image was captured with a 12-megapixel Olympus (DP-70) digital camera. With the aid of home-made software, the damaged CA1 region was encountered and this area was determined. The labeled cells were then counted manually by a person not involved in the experiments, and who knew nothing about the experimental groups. The labeled cells were calculated for 1 mm^2. The numbers of FJ_+ and NeuN-immunopositive neurons/mm^2 were determined in each slide of all animals.

In vitro electrophysiology

The electrophysiological recordings were conducted 10 days after 4VO. The rats (n = 3 x 6) were decapitated, and coronal slices (400 μm) were prepared from the middle part of their hippocampi with a vibratome (Campden Instruments, Serial No: 752-903, UK) in an ice-cold artificial cerebrospinal fluid solution composed of (in mM): 130 NaCl, 3.5 KCl, 1 NaH_2PO_4, 24 $NaHCO_3$, 1 $CaCl_2$, 3 $MgSO_4$, and 10 D-glucose, saturated with 95% O_2 and 5% CO_2. The slices were then transferred into a Haas-type recording chamber and incubated at room temperature for 1 h to allow the slices to recover in the solution used for recording (differing only in that it contained 3 mM $CaCl_2$ and 1.5 mM $MgSO_4$). The flow rate was 1.5-2 ml/min and the experiments were performed at 34 °C. The stimulating electrode (a bipolar glass electrode pulled from a theta capillary) was placed in the stratum radiatum near the border of the CA3 region to perform orthodromic stimulation of the Schaffer collateral/commissural pathway (Accupulser A310 (WPI Inc., USA): constant current, 0.2-ms pulses delivered at 0.033 Hz).

Field excitatory postsynaptic potentials (fEPSPs) were recorded in parallel from the stratum radiatum with two 1-2-MOhm resistance glass microelectrodes that were filled with arteficial cerebrospinal fluid and connected to a neutralized, high-input impedance preamplifier (100x gain) with a high-pass filter set at 5 kHz. A home-made amplifier was used; the sampling rate was 10 kHz. The test stimulus intensity was adjusted to the 30 - 60 μA range to evoke approximately 50% of the maximal stimulus intensity that evoked a saturated fEPSP (maximal fEPSP response) in the SC rats. The fEPSPs were digitized, saved via a PC equipped with a Digidata 1200 interface and an Axoscope10.0 recording system (Molecular Devices Corporation, Sunnyvale, CA, USA), and analyzed off-line with Origin6.0 (OriginLab Corporation, Northampton, MA, USA).

LTP of the Schaffer collateral-CA1 synaptic response was induced by high-frequency stimulation (HFS) (0.2-ms pulses delivered at 100 Hz for 6 s) at 100% intensity of the test stimulus, then the fEPSPs were recorded for a further period of at least 60 min. Input-output (IO) curves were created to measure the basal Glu-ergic synaptic function. Slices from the same animal were generally used for both tests, including LTP and IO curves. Two slices were tested from each rat, and each slice was subjected to only one particular test.

HPLC-MS/MS analysis of KYN and KYNA levels in the plasma and brain tissue

The vertebral arteries were occluded 24 h before treatments with vehicle or KYN and PROB. Two hours later, the rats were anesthetized with pentobarbital (60 mg/kg i.p.). No bilateral carotid artery occlusion was performed. Blood was taken from the aorta, and centrifuged with a Heraeus Megafuge 1.0 R centrifuge (Kendro Laboratory Products GmbH, Osterode, Germany) for 10 min (RCF: 1625 x g). After decapitation, tissue samples were obtained from the hippocampus and cerebral cortex, and weighed. The plasma and brain tissue was stored at about -70 ^{o}C until analysis. Upon thawing, the brain tissue was homogenized in a potter. Calibration curves were prepared by adding variable amounts of KYN and KYNA to the drug-free plasma and the homogenate of the brain tissue. Both the plasma and the homogenized brain tissue were extracted by mixing in a vortex for 30 min, using acetonitrile:distilled water (1:1 by volume). Following centrifugation, the supernatants were evaporated to dryness at 60 ^{o}C, and dissolved in mobile phase A. The HPLC-MS/MS system consisted of a Waters Alliance 2795 HPLC, and a Quattro Ultima Platinum mass spectrometer. Gradient chromatographic separation of KYN and KYNA was achieved with

2.1% formic acid in distilled water (v/v, mobile phase A) and acetonitrile (mobile phase B) on a Purospher STAR RP-8 end-capped column. A linear gradient from 100% A to 60% A was programmed between 0 and 5 min, and then to 0% A between 5 min and 6.5 min. The retention windows (min) of KYN and KYNA were 3-4.3 and 3.8-5.4, respectively, detected by a Waters 2487 UV detector. Ionization was performed in the positive ion mode. The dwell time was set to 200 ms, and the two drugs were monitored in the multiple-reaction monitoring (MRM) mode. The most abundant fragment ions, m/z 209 and 192, were set for KYN, and 209 and 146 for KYNA were selected for detection. The orifice and ring potentials were optimized to the highest signal for each analyte.

Statistical analysis

Neuronal cell counts are presented as means ± S.E.M., and were analyzed by using one-way ANOVA followed by the Bonferroni test for multiple comparisons with SPSS version 9.0 for Windows software (SPSS Inc., Chicago, IL, USA). A $p \leq 0.05$ was considered significant. A nonparametric test on two independent samples was chosen for electrophysiological data (Mann-Whitney U-test).

Results

Histology

In animals subjected to 10-min 4VO, severe neuronal damage was observed in the CA1 area of the hippocampus in both hemispheres 10 days after the intervention. The neuronal damage extended for millimeters in the anterior-posterior direction from -3.14 mm relative to the bregma. In this injured region, numerous of the pyramidal cells in the CA1 region were FJ_+ in each of the coronal sections, while those in the CA3 region and the dentate gyrus were not labeled (Fig. 4A).

In accordance with this, the NeuN immunohistochemistry indicated a lack of intact cells in the CA1 region in the 4VO animals, but an intact CA3 region and dentate gyrus (Fig. 4B).

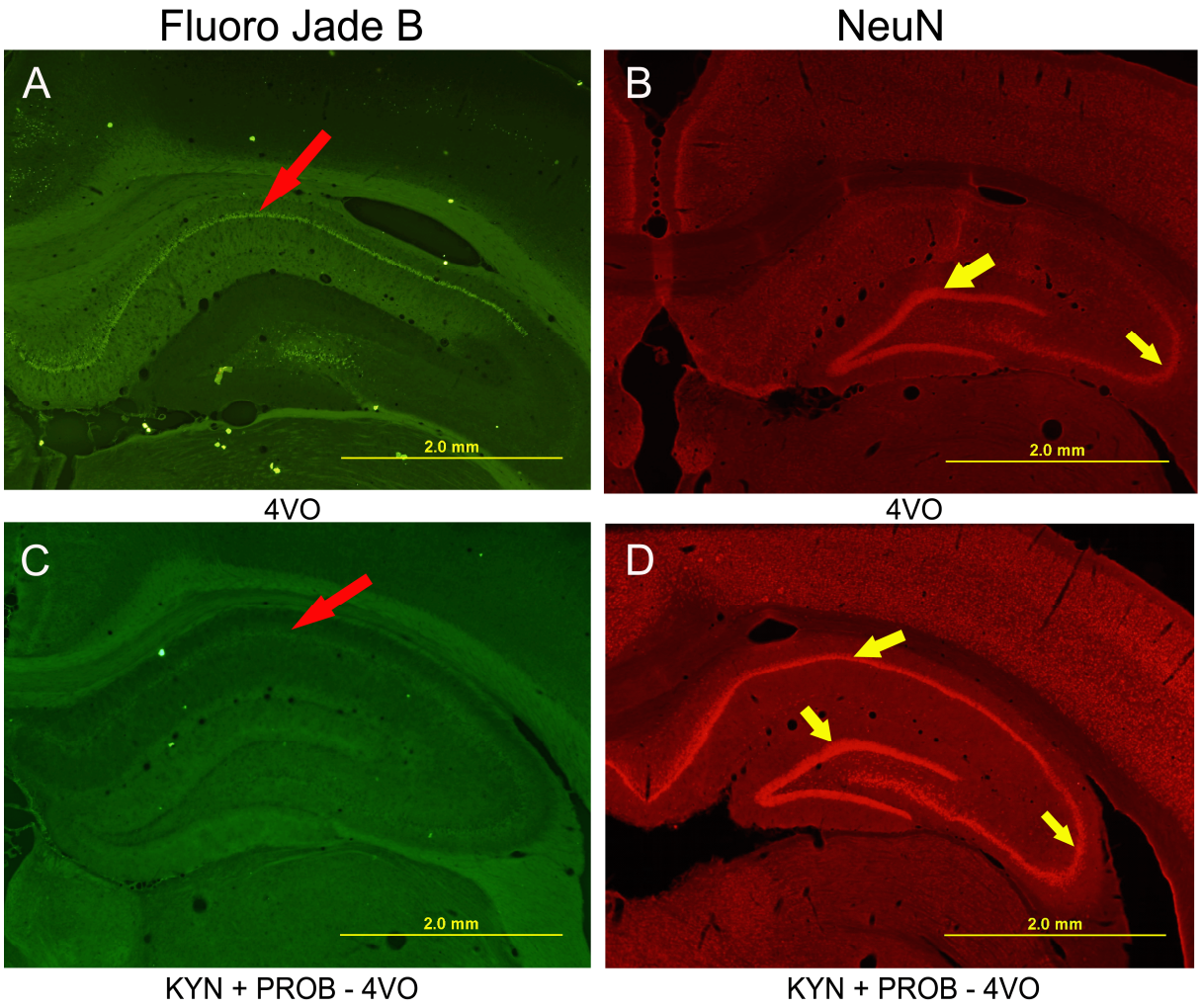

FIGURE 4. The effects of pretreatment with KYN on the ischemic hippocampus of the rat. **A:** Transient global ischemia (4VO)-induced damage of the CA1 pyramidal cells in the hippocampus (the red arrow shows the injured CA1 region (FJ+ cells) of the hippocampus). **B:** The CA3 and dentate gyrus are less vulnerable to transient ischemia: the NeuN-immunopositive region of the hippocampus (yellow arrows). **C:** Pretreatment of 4VO animals with KYN+PROB (KYN+PROB-4VO) protects the hippocampus from the ischemia-induced damage (red arrow). **D:** In (KYN+PROB-4VO)-pretreated rats, NeuN immunohistochemistry reveals intact areas throughout the hippocampus, including the CA1 and CA3 regions and the dentate gyrus (yellow arrows).

KYN administration

KYN (300 mg/kg, i.p.) administered together with PROB (200 mg/kg, i.p.) caused a marked reduction in the number of damaged neurons. In the KYN-pretreated animals, injured neurons stained with FJ-B could be observed only sporadically in the CA1 area of the hippocampus (the same was true for the CA3 region and the dentate gyrus; Fig. 4C).

Accordingly, NeuN immunohistochemistry gave the impression of a non-injured CA1 region (like the CA3 region and the dentate gyrus) in KYN-pretreated 4VO animals (Fig. 4D).

KYN administration considerably decreased the number of injured neurons in the CA1 region. However, the decrease in the number of injured neurons was highly significant only in the pretreated group (KYN+PROB-4VO). The animals in the post-treated group (4VO-KYN-+PROB) also exhibited a tendency to a reduction in the number of injured neurons, but this change was not significant (Fig. 5A). The NeuN immunohistochemistry supplemented these results: the number of non-injured cells was highest in the SC group, and lowest in the 4VO animals. Post-treatment with KYN (4VO-KYN+PROB) had hardly any effect, while in the 4VO animals which received KYN before ischemia (KYN+PROB-4VO) the number of intact cells was comparable to the control level (Fig. 5B).

Fig. 5A

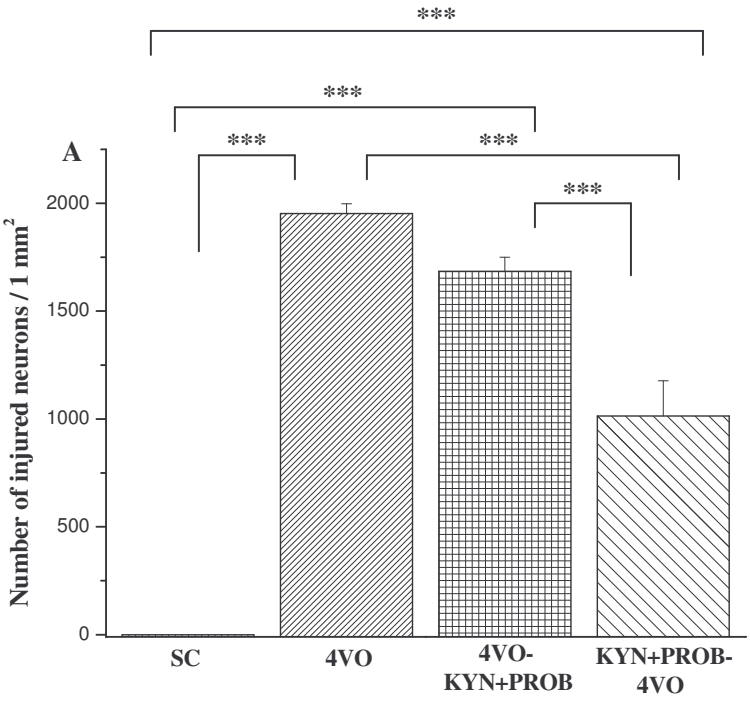

Fig. 5B

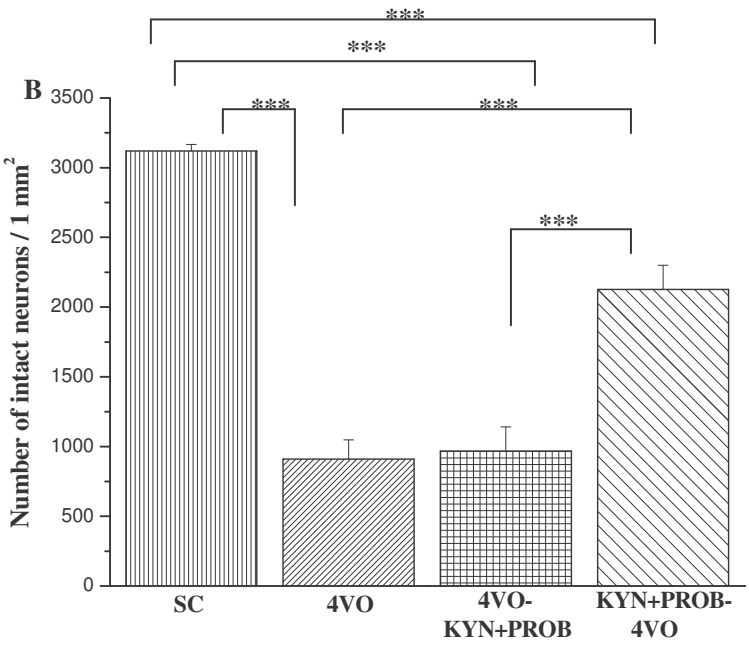

FIGURE 5. The effects of L-KYN administration on the cell number/mm^2 in the CA1 region of four groups of animals.
A: The transient global ischemia (4VO)-induced increase in the number of injured cells was reduced in those animals which received KYN+PROB. The reduction was significant only in those animals which received KYN+PROB before transient global ischemia (KYN+PROB-4VO), though the number of FJ+ cells was slightly altered in the post-treated animals (4VO-KYN+PROB).
B: NeuN immunohistochemistry supplemented the above results: the number of intact cells/mm^2 in the CA1 region of the hippocampus was appreciably reduced in the 4VO animals. Post-treatment (4VO-KYN+PROB) was ineffective, whereas pretreatment (KYN+PROB-4VO) strongly increased the number of intact cells/mm^2 in the CA1 region of the hippocampus. Values are means ± S.E.M. (n = 6-7 rats/group). The differences between the SC and 4VO groups and between the 4VO and KYN+PROB-4VO groups were significant ($p < 0.001$; one-way ANOVA followed by the Bonferroni test).

We also investigated the change in the numbers of injured cells in the cortex (with FJ-B staining). We obtained similar results, *i.e.* a significant neuroprotective effect of L-KYN treatment. The only difference was that post-treatment also proved to be significant in the cortex [59].

In short, the KYN+PROB pretreatment was able to reduce the proportion of damaged cells to 52% relative to the damaged cells induced by 4VO without KYN+PROB treatment.

The KYN+PROB treatment after the 4VO intervention did not prove to be effective in the hippocampus (see Table 1).

Groups:	FJ+ (%):	NeuN-labeled (%):
SC	0	100
4VO	100	29.19
4VO-KYN+PROB	86.29	31.02
KYN+PROB-4VO	51.96	68.17

TABLE 1. The proportions of injured and intact neurons in the CA1 region of the hippocampus. In the case of FJ positivity, the result for the 4VO group was taken as 100%, for the animals in this group exhibited the most extreme injuries. On the other hand, all 4VO-intervention groups were compared with the SC group in the case of NeuN labeling.

In vitro electrophysiology

We explored the basal synaptic properties of the fEPSPs in order to evaluate the ischemia-induced impairment of the Schaffer collateral-CA1 synaptic transmission (Fig. 6).

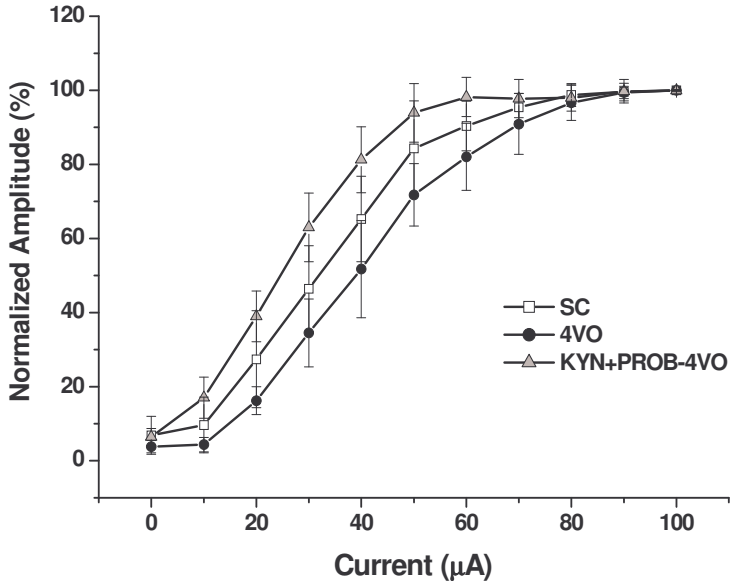

FIGURE 6. IO curves of sham-operated controls (SC), transient global ischemic (4VO) animals, and 4VO animals which received KYN+PROB treatment. IO curves were established by plotting the fEPSP amplitudes against various test pulse intensities from 0 to 100 μA. No significant difference was found between the IO curves in the three groups, implying that the basal functions of the pyramidal cells and synapses were not affected by complete ischemia.

For this purpose, the IO curves were established by plotting the fEPSP amplitudes against various test pulse intensities from 0 to 100 μA. The IO curve for the 4VO animals was positioned below that for the controls (while the curve for the KYN+PROB-4VO animals was

positioned above it). However, there was no significant difference between the IO curves in the three groups, implying that the basal functions of the registered pyramidal cells and synapses were not affected by complete ischemia.

LTP was induced by HFS of the Schaffer collateral-CA1 synapses. The fEPSPs were monitored for 40-60 min before conditioning stimulation until the amplitudes were generally stable, and their mean value was determined as the 20-min-long baseline before LTP induction. In the SC group, the HFS caused a robust increase (170-180%) in the slope of the fEPSPs (Fig. 7, SC) and this increase in slope (and in amplitude) remained at the elevated level during the 1-h registration period. The same conditioning protocol did not induce a significant, lasting increase of the fEPSPs in the majority of the 4VO animals. In this group, the elevation of the amplitudes was only transient; no LTP was observed. At the end of the registration period, the slopes had returned to the control level, or decreased below the baseline (Fig. 7). The administration of KYN and PROB protected slices from the 4VO-induced LTP impairment. KYN restored the fEPSP slopes to the control level, and these parameters were stable until 60 min after HFS.

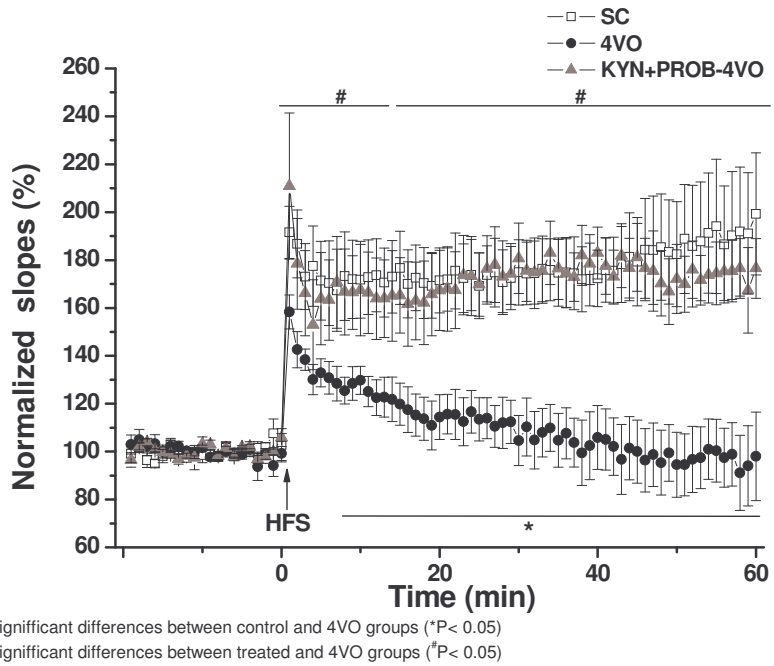

FIGURE 7. Administration of KYN and PROB prevented the impairment of the LTP response caused by global ischemia. In the SC group (n = 5), LTP was induced by HFS of the Schaffer collaterals (600 pulses at 100 Hz). LTP after HFS was not present in the 4VO group (n = 11) 10 days after 10-min global cerebral ischemia. The KYN+PROB-4VO group (n =6) was subjected to the same carotid occlusion that was preceded and followed by i.p. injections

of KYN and PROB. The course of LTP in this group was identical to that in the controls. Data points are means ± S.E.M. of normalized slopes of fEPSPs.
Statistically significant differences are denoted: (*), (#) significant differences between SC and 4VO groups (*$p < 0.05$), and between treated and 4VO groups (#$p < 0.05$; a nonparametric test on two independent samples was used, the Mann-Whitney U-test).

Plasma and brain KYN and KYNA concentrations

In the sham-operated rats, the KYN concentration was approximately 3-fold higher in the plasma than in the cortex and hippocampus. On the other hand, the KYNA concentration was higher in the cortex and hippocampus than in the plasma ($p < 0.001$), and higher in the cortex than in the hippocampus. Treatment with KYN+PROB considerably increased both the KYN and KYNA concentrations in the plasma and brain, and also altered their proportions within the compartments studied. The KYN concentration increased 37-fold in the plasma and approximately 70-fold in the hippocampus and cortex, while the KYNA concentration was elevated roughly 300-fold in the plasma and 50-fold in the hippocampus and cortex. The KYN concentration increased more in the brain than in the plasma, and the KYN levels were similar in the plasma and brain after KYN+PROB administration, but this was probably due to the relatively large scatter of the data. Likewise, since the KYNA concentration increased more in the plasma than in the brain, the KYNA concentrations were similar in the plasma and brain, reaching 7-12% of the KYN concentration in the brain (Table 2).

	Plasma concentration, ng/ml		Hippocampal concentration, ng/g wet weight		Cortical concentration, ng/g wet weight	
	KYN	KYNA	KYN	KYNA	KYN	KYNA
Vehicle, *n* = 5	602 ± 21	8.8±0.5	212±18***	17.7±2.1*** #	235±22	28.2±3.2 ***
KYN + PROB, *n* = 5	22518 ±5392	2695±783	14270±3801	996±318	17101±4492	1497±534
Ratio treated/sham	37	305	67	56	73	53

TABLE 2. The concentrations of KYN and KYNA in the plasma, brain cortex and hippocampus in rats in which the vertebral arteries were occluded bilaterally on the day before treatment. The animals were treated with vehicle or KYN + PROB 2 h before sacrifice, but no bilateral carotid artery occlusion was performed.
Statistically significant differences are shown: *** $p < 0.001$ vs plasma; # $p < 0.05$ vs cortex.

Discussion

Evidence is accumulating which suggests that an ischemia-induced sustained elevation in intracellular calcium concentration $[Ca^{2+}]_I$ contributes to cell death[24,32]. The secondary rise during reperfusion injury is more likely to be responsible for apoptotic than for immediate necrotic damage, which occurs in the CA1 neurons in particular 2-3 days after the induction of global ischemia[68]. These processes of injury of the CA1 neurons are attained via NMDA activation[29].

One promising neuroprotective intervention might involve modulation of the KYN pathway. Accordingly, the systemic administration of KYN dose-dependently increases the KYNA content of the brain[81]. The normal nanomolar concentration of KYNA in the brain[42] can be elevated 1000-1300-fold following peripheral administration of KYN+PROB, a known inhibitor of the transport of organic acids, including KYNA[40,81]. KYN administration has proved to be neuroprotective in histological studies. However, the neuronal degeneration marker used, FJ-B, does not discriminate between apoptotic and necrotic cell damage. Moreover, recent studies have indicated that FJ-B can stain neurons degenerating as a result of an acute insult, and it can label activated microglia and astrocytes during a chronic neuronal degenerative process[13,23]. All these points should be taken into consideration. The systemic administration of KYN together with PROB resulted in concentrations of KYNA in the brain which have proved to be neuroprotective in histological and behavioral studies. However, relatively little is known about its impact on the outcome of synaptic plasticity. A novel finding here is that the administration of KYN (+PROB) once before and 4 times after 4VO-induced transient global ischemia proved neuroprotective in histological studies, and also reduced (nearly abolished) the impaired LTP induction in the Schaffer collateral-CA1 pathway in adult rats. Interestingly, the IO curves of the controls, and of ischemic and of ischemic+KYN+PROB-treated rats did not display significant changes. This suggests that it is the machinery of LTP that is injured rather than the basal functions of the pyramidal cells and synapses. It should additionally be taken into consideration that, though the FJ-B labeling indicates massive neuronal degeneration in the untreated 4VO group, the NeuN positivity demonstrates that nearly a third of the neurons in this group remained intact, in spite of the complete ischemia. Though we do not know sufficient about ischemia-induced injuries of the LTP machinery, it is clear that ischemia may impair physiological forms of synaptic

plasticity, such as activity-dependent LTP[19]. Our experiments detected fEPSPs with increased amplitudes and an elevated potentiation ability in only one 4VO animal (not presented). In most cases, however, after transient global ischemia, the surviving neurons displayed normal transmission, except for the reduction in the maximum levels of fEPSPs that seems to be a consequence of the cell number reduction due to ischemic cell death. The impaired LTP induction should reflect deficits in the machinery specific to LTP induction in the individual surviving neurons. Although the mechanism is not yet known, we have demonstrated for the first time here that treatment with KYN (and PROB) rescues the Schaffer collateral-CA1 synapses from impaired LTP induction after transient global ischemia.

The results cited above and our own observations presented here suggest that KYN administration results in the production of the neuroprotective KYNA rather than the neurotoxic QUIN. This assumption is supported by the present complex histological and electrophysiological results, which prove that KYN, as a precursor of the Glu receptor antagonist KYNA, offers advantages over KYNA in the treatment of ischemic brain damage.

These results raise the possibility that long-term KYN administration may be useful in delaying neuronal loss in certain neurodegenerative disorders. However, one should not be over-hasty in drawing facile conclusions: first, because only the pretreatment was protective, and second, because KYN treatment may give rise to serious side-effects on the Glu neurotransmission.

EXPERIMENT 2

EFFECTS OF SYSTEMIC ADMINISTRATION OF L-KYN ON THE CORTICOCEREBRAL BLOOD FLOW UNDER NORMAL AND ISCHEMIC CONDITIONS OF THE BRAIN IN AWAKE RABBITS

The aim of the present study was to examine whether peripherally administered L-KYN can influence the normal and the unilateral carotid artery occlusion-induced ischemic cCBF in awake rabbits (Sas et al., 2003). Csete et coworkers[12] earlier elaborated a method in rabbits which mimicks the cerebrovascular disturbances associated with chronic cerebral ischemia, and allow study of the CBF changes following the ischemic insult.

Materials and Methods

Animals

Altogether 60 New-Zealand white rabbits weighing 2.5-3 kg were used. The animals were fed commercial laboratory rabbit food pellets and had free access to water.

Surgical preparation

Introduction of electrodes into the cerebral cortex. Rabbits were anesthetized with an intravenous solution of diazepam (20 mg/kg) and ketamine (20 mg/kg) in physiological saline solution. Six hydrogen-sensitive electrodes, consisting of glass-insulated (O.D. 0.5 mm) platinum wire (diameter 0.1 mm) with a bare tip length of 1 mm, were inserted stereotaxically into the parietal cortex at the following coordinates: 5 mm from the sutura sagittalis, 2-12 mm from the bregma, and at a depth of 5 mm from the horizontal plane passing through the bregma on both sides, through adequately placed bore holes in the skull, and fixed with dental cement under aseptic conditions. Stereotaxic placements were calculated from the atlas of Monnier-Gangloff [41]. Before wound closure, antibiotic Carbenicillin (as powder) was applied locally. After a 3-5-day recovery period, the animals were in good physical condition.

Occlusion of the carotid artery. In the animals in the ischemic group, permanent surgical occlusion was accomplished with ligatures on the left external and internal carotid arteries. The operation was performed under the same general anesthesia as above.

Measurements

Measurement of cCBF. The rabbits were allowed to recover for 3 days after surgery, and the cCBF was determined at 6 sites in their brain by means of the hydrogen clearance technique[2].

During the measurements, the rabbits were resting quietly in a comfortable wooden stock and 2-5% hydrogene gas was administered via a funnel. Clearance curves were registered and the cCBF was evaluated with the aid of an 80 MHz IBM 486 SX computer.

The basal and the unilateral carotid occlusion-induced impaired cCBF, and the flow changes following drug treatment were determined. One of the groups consisting of 6 control awake rabbits without drug treatment was used to measure the effect of physiological saline solution (in the same volume as used for drug treatments), as control, on the cCBF.

Measurement of MABP, HR and blood gas parameters. The MABP was measured in awake rabbits via a catheter inserted into the left or right central ear artery, connected through a pressure transducer (Statham p23 Dp) to a Hellige electromanometer. At the same time, the ECG was continuously recorded by a radiotelemetry system in order to monitor the HR. Two electrodes were placed on both sides of the shaven chest of the rabbits, near to the heart. The ECG was registered via radio waves from the animal to a receiver (Innopoint, Budapest), connected through an ECG telemonitor (MOD-DEM, Kell-78-04, Medicor, Hungary) to a three-channel recorder (BIOSET 3000; Medicor). Arterial pH, pCO_2, pO_2 and O_2 saturation were measured at intervals, utilizing a blood gas analyzer (Model OP-216, Radelkis, Budapest).

Drug treatment

The marginal ear vein was cannulated and used for the administration of L-KYN and other agents.

L-KYN free base (Sigma, St. Louis, MO, U.S.A.) was freshly dissolved in physiological saline solution each day and was administered intravenously in a volume of 1.5-2 ml. First the baseline values were registered, and then the effects of L-KYN on the cCBF, MABP and HR were measured for 240 min.

As vasodilation in the cerebral vessels can be mediated via acethylcholine and/or nitric oxide (NO), we gave their selective inhibitors to investigate the potential mechanisms of L-KYN on cerebral vessels.

Atropine sulfate (1 mg/kg) (EGIS Rt., Hungary) was given intravenously in aqueous solution in a volume of 2-3 ml 5 min before the administration of L-KYN (1 mg/kg), and the cCBF, MABP and HR values and the L-KYN-induced responses were registered.

Nitro-L-arginine methyl ester (L-NAME) (40 mg/kg) (Sigma, St Louis, MO, USA) was given intravenously in aqueous solution in a volume of 1 ml after registration of the basal cCBF values. Thereafter, the measurements were repeated; 45 min later L-KYN (1 mg/kg) was administered and the changes in cCBF, MABP and HR were determined. The dose of L-NAME was chosen on the basis of the results of Iadecola et al.[24] according to which 40 mg/kg of the compound was effective in attenuating the hypercapnic cCBF response, reaching a maximum at 45 min.

Statistical analysis

The results were expressed as means ± SEM. Data were collected in pairs from the same measuring sites (electrodes) before and after the experimental intervention or the administration of agents. The statistical significance of the observed differences was calculated by Student's paired *t*-test and repeated measures analysis of variance (ANOVA). Multiple comparison of different time points and groups was carried out by means of one-way ANOVA. p values < 0.05, when obtained with both statistical tests, were considered significant.

Significance of reductions in mean basal cCBF values in the controls and in animals with unilateral carotid occlusions treated with 0.3, 1 and 3 mg/kg L-KYN (Group "1", "2" and "3") were calculated by means of the unpaired Student's *t*-test. p values < 0.05 were considered significant.

Results

Administration of L-KYN resulted in a significant increase in the normal cCBF (Table 3). This effect was particularly obvious after the administration of 1 mg/kg L-KYN. The cCBF was enhanced by all three applied doses of L-KYN within 30 min, attained its maximum values soon thereafter, and remained high even around the 240th min, *i.e.* at the end of the recording period.

TIME (min)	L-KYNURENINE (mg/kg i.v.) 0.3		1		3	
	cCBF (ml/min/100 g tissue)					
0 (basal value)	**117±15**	**%**	**90±12**	**%**	**113±10**	**%**
1	133 ± 19	14	81 ± 90	-10	118 ± 12	4
30	130 ± 14	11	104 ± 18*	16	159 ± 21*	41
60	137 ± 19	17	106 ± 13*	18	147 ± 16*	30
90	142 ± 22*	21	136 ± 19*	51	153 ± 20*	35
150	135 ± 20	15	168 ± 26*	87	157 ± 15*	39
180	125 ± 14	7	146 ± 27*	62	153 ± 17*	35
210	155 ± 24*	32	145 ± 24*	32	148 ± 17*	31
240	163 ± 22*	39	137 ± 30*	32	163 ± 27*	44
N	*20*		*20*		*20*	

TABLE 3. Effects of L-KYN on cCBF in control awake rabbits. Means ± SEM, *n* = total number of measurements (electrodes) in each group of 6 rabbits. *p < 0.05 vs basal value at 0 min, % change from the basal value.

Treatment with physiological saline solution did not change the cCBF significantly. The following cCBF values (ml/min/100 g tissue; mean ± SEM) were measured: baseline=107±15; 30min=123±12; 60min=108±14; 90min=117±11; 120min=103±16; 150min=104±15, 180min=114±16; 210min=106±12; 240min=110±11.

Unilateral carotid occlusion caused a significant reduction in cCBF from 117±15 to 57±11 (“1”), from 90±12 to 52±8 (“2”) and from 113±10 to 58±11 (“3”) ml/min/100 g tissue (Fig. 8).

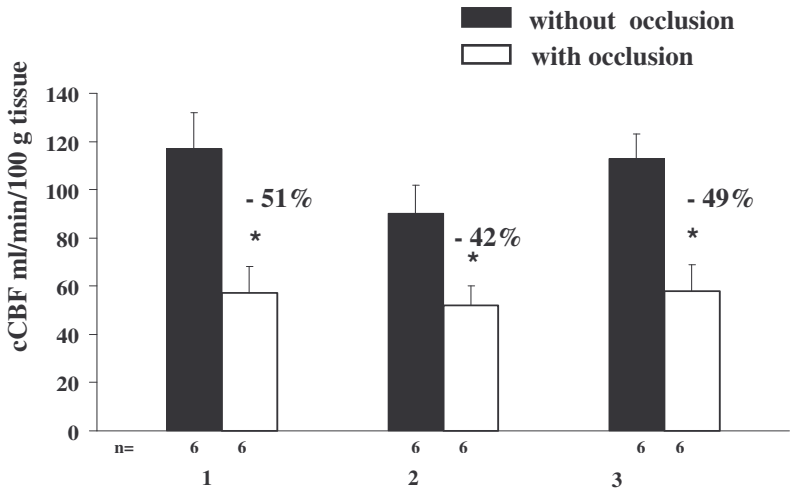

FIGURE 8. Unilateral carotid occlusion-induced cCBF reduction in 3 different groups of rabbits.

Following the administration of L-KYN, there was an immediate increase in the cCBF in the animals with carotid occlusion (Table 4). The L-KYN-induced maximal percentage increases in the cCBF in the ischemic animals were more pronounced than the cCBF decreases caused by unilateral occlusion (92-94% /at 1 mg/kg dose/ *vs* 42-51%) (Fig. 8, Table 4), and in the L-KYN-treated animals with carotid occlusion, the cCBF became closer to or even exceeded (at 1 mg/kg L-KYN) the normal basal values measured in the control animals (Tables 3 and 4). This effect was of long duration, and peak values were recorded at 60-240 min after L-KYN injection.

TIME (min)	L-KYNURENINE (mg/kg i.v.) 0.3		1		3	
	cCBF (ml/min/100 g tissue)					
0 (basal value)	57 ± 11	%	52 ± 80	%	58 ± 11	%
1	61 ± 14	7	65 ± 90	25	66 ± 10	14
30	69 ± 11	21	80 ± 11*	54	81 ± 12*	40
60	72 ± 15	26	101 ± 14*	94	94 ± 13*	62
90	78 ± 13*	37	84 ± 11*	62	91 ± 13*	57
120	85 ± 17*	49	100 ± 12*	92	97 ± 12*	67
150	86 ± 14*	51	76 ± 8*	46	103 ± 12*	78
180	77 ± 11*	35	87 ± 10*	67	93 ± 11*	60
210	73 ± 15*	28	96 ± 15*	85	99 ± 15*	71
240	87 ± 15*	53	99 ± 17*	90	95 ± 13*	64
N	*20*		*20*		*20*	

TABLE 4. Effects of L-KYN on cCBF in awake rabbits with unilateral carotid occlusion. Means ± SEM. *n* = total number of measurements (electrodes) in each group of 6 rabbits. **p* < 0.05 *vs* basal value at 0 min, % change from the basal value.

Pretreatment with atropine or L-NAME prevented the cCBF-increasing effect of 1 mg/kg L-KYN in the control rabbits and also in those with carotid occlusion (Table 5).

	cCBF ml/min/100 g tissue									
	L-KYNURENINE (1 mg/kg i.v.)									
	time (min)									
DRUG	0 (basal value)	1	30	60	90	120	150	180	210	240
Atropine Sulfate 1 mg/kg i.v.										
Control *n*=20	91±6	81±70	88±8	74±11	74±11	74±70*	66±60*	68+7*	69±70*	66±60*
with carotid occlusion *n*=20	46±7	42±60	51±80	44±70	42±60	43±60	41±5	42±50	42±60	42±50
L-NAME 40 mg/kg i.v.										
Control *n*=20	90±18	88±19	80±17	60±10*	70±16*	71±12*	58±10*	61±15*	64±18*	62±16*
with carotid occlusion *n*=20	53±40	51±40	55±80	57± 90	68±17	55±70	52±5	50±5	51±50	52±60

TABLE 5. Effects of atropine or L-NAME pretreatment on KYN-induced changes in cCBF in awake rabbits. Values are means ± SEM. *n* = number of measurements (electrodes) in each group of 6 rabbits. **p* < 0.05 *vs* basal value at 0 min.

As shown in Table 6, L-KYN did not alter the MABP or HR, whereas L-NAME caused a small increase in the MABP as did atropine in the HR. The duration of the latter effects was relatively short (< 60 min).

		MABP (mmHg)		**HR** (beats/min)	
Groups/ Doses (mg/kg)	*n*	Baseline	Treated	Baseline	Treated
Control					
L-KYN					
0.3	6	100 ± 2	102 ± 4	230 ± 4	231 ± 8
1	6	101 ± 3	100 ± 4	225 ± 5	230 ± 6
3	6	105 ± 2	106 ± 3	227 ± 6	235 ± 5
Atropine					
1	6	95 ± 5	100 ± 3	224 ± 5	240 ± 6
L-NAME					
40	6	90 ± 2	110 ± 2 *	220 ± 6	235 ± 5
With carotid occlusion					
L-KYN					
0.3	6	105 ± 3	107 ± 4	230 ± 5	227 ± 4
1	6	100 ± 5	105 ± 6	240 ± 5	238 ± 3
3	6	98 ± 4	102 ± 2	228 ± 7	235 ± 4
Atropine					
1	6	105 ± 5	107 ± 6	225 ± 5	242 ± 7
L-NAME					
40	6	102 ± 4	125 ± 3*	235 ± 5	245 ± 4

TABLE 6. Effects of L-KYN, atropine and L-NAME on MABP and HR in awake rabbits. Mean maximum values ± SEM are given. *n* = number of animals. * $p < 0.05$ *vs* baseline value. MABP: mean arterial blood pressure; HR: heart rate.

No significant changes in the arterial blood gas parameters or pH were noted after the administration of L-KYN, atropine or L-NAME (Table 7).

Groups/doses (mg/kg)	*n*	pH	pO_2 (mmHg)	pCO_2 (mmHg)	O_2 saturation
Control					
Baseline	6	7.47 ± 0.04	95 ± 2	34 ± 0.6	98 ± 0.4
L-KYN					
0.3	6	7.45 ± 0.05	98 ± 1	36 ± 0.8	98 ± 0.2
1	6	7.44 ± 0.01	94 ± 2	30 ± 2	97 ± 1
3	6	7.36 ± 0.02	93 ± 2	31 ± 2	98 ± 1
Atropine					
1	6	7.38 ± 0.03	96 ± 1	33 ± 2	100 ± 2
L-NAME					
40	6	7.42 ± 0.02	96 ± 2	31 ± 2	98 ± 1
With carotid occlusion					
Baseline	6	7.46 ± 0.02	95 ± 2	33 ± 1	97 ± 2
L-KYN					
0.3	6	7.46 ± 0.01	97 ± 1	34 ± 2	98 ± 1
1	6	7.43 ± 0.02	98 ± 2	31 ± 1	98 ± 1
3	6	7.45 ± 0.03	94 ± 3	32 ± 3	96 ± 3
Atropine					
1	6	7.44 ± 0.02	96 ± 1	35 ± 2	97 ± 1
L-NAME					
40	6	7.42 ± 0.01	96 ± 1	31 ± 1	98 ± 2

TABLE 7. Arterial blood parameters in awake rabbits after the administration of L-KYN, atropine or L-NAME with or without unilateral carotid occlusion. Mean maximum values ± SEM are given, *n* = number of animals.

Discussion

Relatively little information is available concerning the effects of the KYN system on the CBF. We have investigated the effects of L-KYN on the cCBF, as measured by means of the hydrogen clearance technique. The changes in cCBF following intravenous L-KYN administration were measured at 1 min and then every 30 min up to 4 h in awake rabbits with or without prior exposure to unilateral carotid artery occlusion. These measurements were performed 3 days after the implantation of the electrodes and occlusion of the carotid artery. Peripherally administered L-KYN is known to cross the BBB easily and to reach maximal cerebral concentrations within 60-120 min[81]. The administration of L-KYN resulted in significant and immediate increases in cCBF in both animal groups, either with or without carotid occlusion. During the experimental period, the parameters of the systemic circulation

remained unchanged. The pronounced CBF increase in animals with carotid occlusion is quite noteworthy since other compensatory mechanisms producing vasodilation must have already been in action. L-KYN was effective in doses as low as 0.3-1 mg/kg. The overall improvement in cCBF was more pronounced in animals with unilateral carotid occlusion. In the control group, the highest elevation occurred at a dose of 1 mg/kg dose (87%). In the animals with chronic cerebral ischemia, a significant improvement in cCBF was seen at all doses, with maximal increases at doses of 1 and 3 mg/kg doses (94% and 78%, respectively).

Pretreatment with atropine or the general NOS inhibitor L-NAME prevented the increase in cCBF following L-KYN treatment in both groups.

The mechanism whereby an elevated cerebral L-KYN level leads to an increase in cCBF has not been elucidated. Few data are available concerning the possible vasoactive potential of KYNA or QUIN, the two main metabolites of L-KYN.

In contrast with our findings, no changes in vessel diameter were found in anesthetized newborn piglets under normal condition and after 10 min of global cerebral ischemia induced by the topical administration of either KYNA or QUIN (both at 10^{-5} to 10^{-3} M) measured by the closed cranial window technique[4]. Interestingly, another NMDA receptor antagonist, CGS-19755, also enhanced the cCBF in rats with occluded left middle cerebral and common carotid arteries, when given in a dose of 15 mg/kg immediately after the occlusions[76]. MK-801 had a similar effect in normal rats[66], although in another experiment, where measurements were made under halothane anesthesia, MK-801 significantly reduced the local cCBF in the majority of the brain regions of rats in both the ischemic and non-ischemic hemispheres[51]. A potential explanation for this latter finding is that the functional consequences of the blockade of the NMDA receptors are markedly modified by anesthetic agents[28]. The reason for the increase in cCBF may be the activation of the ascending cholinergic pathways as a response to the NMDA receptor blockade[36]. Acetylcholine is a potent vasodilator in the central nervous system. Ketamine (1 mg/kg), an open channel blocker of the NMDA receptor, also enhanced the cCBF in halothane-anesthetized rabbits[47]. In another experiment, the increase in cCBF caused by 1 mg/kg ketamine could be blocked by another anticholinergic drug, scopolamine hydrobromide (2 mg/kg), and it was concluded that ketamine activates a cholinergic vasodilator system[57]. In our study, coadministration of 1 mg/kg L-KYN with 1 mg/kg atropine sulfate prevented the effect of KYN, but it is

questionable whether its mechanism is similar to that mentioned above. The human brain extracellular KYNA concentration is about 100 nM[62]. To exert pharmacological effects, *i.e.* an antagonism at the Gly allosteric site of the NMDA receptor, micromolar concentraions of KYNA are necessary[5]. It's questionable wether systemic administration of L-KYN in a dose as low as 1 mg/kg in our study, causes NMDA receptor antagonism at all. Studies do not appear to have been reported in which such small doses were used *in vivo* to investigate NMDA receptor binding.

We earlier found that preischemic application of KYN attenuated the infarction size in normotensive mice and improved the neurological outcome in gerbils[18]. However, KYNA proved to be ineffective in reducing the infarct size in spontaneously hypertensive rats, which have an impaired collateral circulation[61]. This raises the possibility that KYNA may exert its neuroprotective effect not only by inhibiting excitatory neurotransmission, but also by increasing the (collateral) blood flow.

The concentrations of QUIN decreased or remained unchanged in gerbils during the first 24 h after ischemia, but were significantly increased at 2 days and even higher after 4 and 7 days[21]. This period following ischemia is often characterized by an increased blood flow. It does not seem likely, however, that the low dose of L-KYN used in our experiment could significantly enhance the brain QUIN content.

A direct vasodilatory effect of L-KYN or one of its derivatives on cerebral vessels is doubtful.

A role for NO in producing the enhancement in cCBF can be presumed. In our study, the CBF-increasing effect of L-KYN was blocked by L-NAME. NO can be induced by short-term cerebral ischemia via NMDA receptor activation or interestingly, as a result of NMDA receptor blockade-induced increased parasympathetic activity[48]. NMDA receptor activation-induced NO production could be attenuated by pretreatment with ketamine (50 mg/kg), an open channel NMDA receptor blocker[31].

Glu, NMDA and kainate have been shown to dilate cerebral arterioles in animals, while in contrast with the above findings, MK-801, a potent inhibitor of NMDA receptors, completely blocks this[3,39], and NO synthase inhibitors attenuate Glu-induced cerebral vasodilation[35]. These findings suggest that the vasodilatory response is mediated by NO through NMDA receptor activation. NMDA receptor stimulation in cortical neurons results in

calcium influx, membrane depolarization, activation of intracellular nNOS, and the subsequent production of NO, which diffuses to the cerebral arteries and causes relaxation of the vascular smooth muscle without the involvement of astroglia or endothelium[10]. Thus, it may be the neuronal source of NO which plays a role in this effect.

It is apparent therefore, that the mechanism by which L-KYN or its derivatives increases the cCBF remains to be clarified (reviewed by Sztriha, Sas and Vécsei[75]). Whatever the precise mechanism is, besides the known neuroprotective action, if the systemic administration of L-KYN results in an additional beneficial vasoactive effect, it may provide a useful alternative in the therapy of cerebrovascular disorders. Further studies are needed to define the mode of action of L-KYN as a vasodilatory agent.

EXPERIMENT 3
EFFECTS OF CITALOPRAM AND FLUOXETINE ON THE CORTICOCEREBRAL BLOOD FLOW IN AWAKE RABBITS

In our study, we investigated for the first time the effects of two SSRI antidepressants, citalopram and fluoxetine, on the normal and the carotid ligation-induced ischemic cCBF, and also on the MABP and HR in awake rabbits (Sas et al., 2007a).

Materials and Methods

Male and female New Zealand White rabbits (altogether *n*=64) weighing 2.5-3.0 kg were used. The animals were fed commercial laboratory rabbit food pellets and had free access to water.

Surgical preparation

Introduction of electrodes into the cerebral cortex, occlusion of the carotid artery: This was performed in the same way as described in Experiment 2.

Measurements

Measurement of cCBF, MABP and HR: This was also the same as described previously.

Drug treatment

The marginal ear vein was cannulated and used for the administration of drugs, which were freshly dissolved in physiological saline solution each day. The effects of each of the individual doses (0.1, 0.3 and 1.0 mg/kg) of fluoxetine or citalopram were investigated separately and each animal was treated with only one dose of drug. The amounts of drugs applied in the study correspond to the therapeutically meaningful (antidepressant) human doses.

Fluoxetine ((±)-N-methyl-χ-(4-(trifluoromethyl)phenoxy)benzenepropanamine) hydrochloride (Sigma Chemical Co) was administered intravenously as a bolus, in a total volume of 1 ml.

Citalopram hydrochloride (Lundbeck, Kopenhagen) was infused continuously for 60 min (total volume 10 ml).

The basal and the unilateral carotid occlusion-induced impaired cCBF and the cCBF changes following fluoxetine or citalopram treatment were determined at 10 min and every 30 min during 150 min after drug administration.

Statistical analysis

The results were expressed as means ± SEM. Every single implanted corticocerebral electrode was marked throughout the duration of the measurements and served as its own control. Data were therefore collected in pairs from the same measuring sites (electrodes) before and after the experimental intervention or agent administration. Student's paired *t*-test was used to determine whether the difference between pair members was significant. Multiple comparisons of different time points and groups were carried out by means of one-way ANOVA. Only p values < 0.05 with both statistical tests were considered significant.

This work was carried out under the conditions specified in the Guidelines of the Committee on Animal Research, University of Szeged, Hungary, which complies with the European Community Guidelines for the use of experimental animals.

Results

As shown in Fig. 9A,B, fluoxetine and citalopram caused only minor, insignificant changes in the normal cCBF. Unilateral carotid occlusion produced a significant decrease in cCBF in all 6 control groups /from 90±17 to 48±16; from 88±16 to 49±14; from 87±14 to 41±16 (Fig. 9C) and from 92±18 to 48±17; from 97±14 to 56±15; from 98±16 to 55±17 ml/min/100 g tissue (Fig. 9D)/. Fluoxetine did not influence the cCBF significantly at any dose in the ischemic animals; there was only a very slight tendency to an increase (Fig. 9C). In contrast, citalopram (0.1, 0.3 or 1 mg/kg in infusion) improved the unilateral carotid occlusion-induced impaired cCBF response as compared with the basal occluded values. The effect was dose-dependent; the most pronounced change was seen in response to 1 mg/kg citalopram, *i.e.* the highest applied dose of this drug (Fig. 9D). The average maximal changes were: 48±17 → 58±19; 56±15 → 72±19; 55±17 → 82±21 ml/min/100 g tissue at 0.1, 0.3 and 1.0 mg/kg citalopram, respectively.

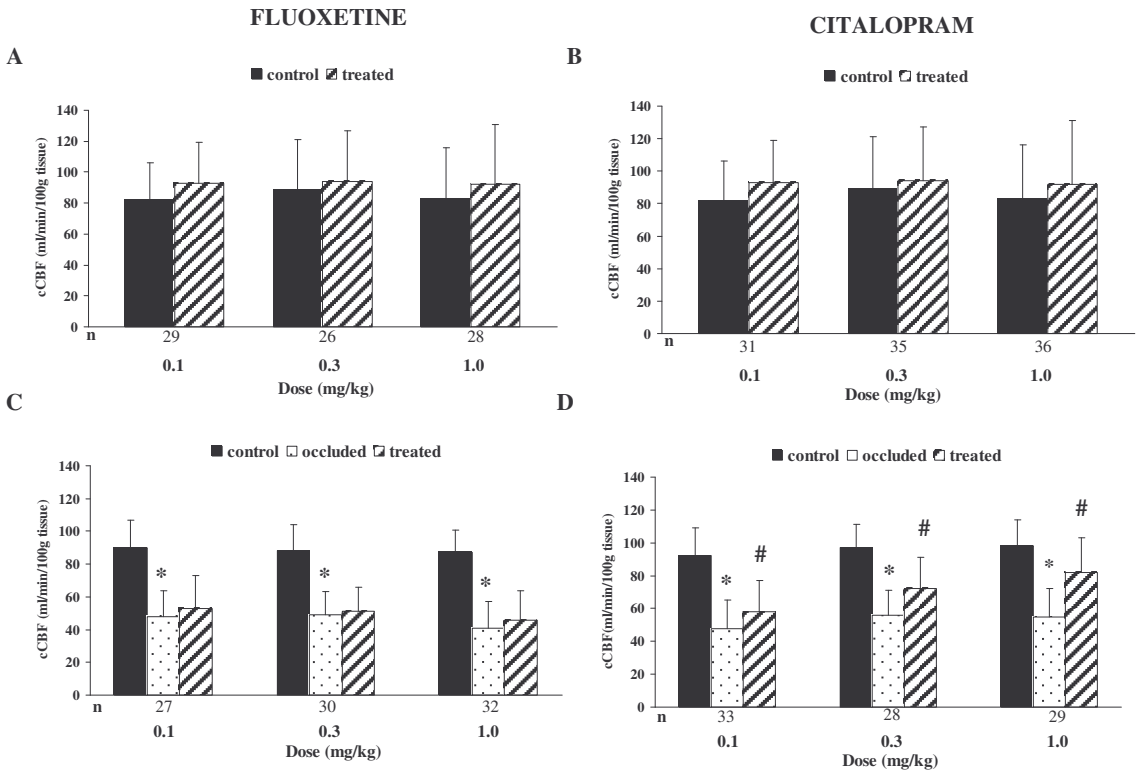

FIGURE 9. Effects of fluoxetine and citalopram on normal and unilateral carotid occlusion-induced cCBF responses. Values are means ± SEM. *n* = number of measurements (electrodes) in 6 animals. Statistical significances were calculated in comparison with the unoccluded control ($^{*}p < 0.05$) and occluded ($^{\#}p < 0.05$) values.

The cCBF-increasing effects appeared toward the end of the 150-min recording period: the peak values were obtained at 90-120 min.

Administration of 10 ml physiological saline solution did not significantly alter the cCBF in either the control or the ischemic group (data not shown).

Both fluoxetine and citalopram decreased the HR as compared with the baseline value. Fluoxetine administration caused a slight, but significant dose-dependent reduction in HR in both the normal and the ischemic group. At the highest dose (*i.e.* 1 mg/kg), the HR was reduced by 13-14% (Table 8). The treatment with citalopram affected the HR significantly only at the 0.3 and 1 mg/kg doses of the drug and the percentage changes were lower relative to those observed after fluoxetine administration (3-5%).

No significant changes in MABP were noted in the fluoxetine-treated groups. However, significant reductions in MABP were observed in the groups treated with 0.3 or 1 mg/kg

citalopram under either normal or unilateral carotid occlusion-induced ischemic conditions (Table 8).

Groups/Doses (mg/kg i.v.)	*n*	**MABP** (mmHg) Baseline	Treated	**HR** (beats/min) Baseline	Treated
Control					
FLUOXETINE					
0.1	6	108 ± 2	111 ± 3	240 ± 3	230 ± 2*
0.3	6	103 ± 1	105 ± 2	238 ± 5	220 ± 3*
1	6	104 ± 3	106 ± 4	230 ± 6	200 ± 5*
CITALOPRAM					
0.1	6	112 ± 5	107 ± 5	250 ± 1	247 ± 2
0.3	6	101 ± 2	92 ± 1*	238 ± 4	231 ± 2
1	6	105 ± 4	89 ± 2*	237 ± 2	225 ± 2*
With carotid occlusion					
FLUOXETINE					
0.1	6	104 ± 3	106 ± 4	230 ± 3	220 ± 2*
0.3	6	102 ± 5	105 ± 6	240 ± 3	228 ± 3*
1	6	101 ± 4	103 ± 2	238 ± 4	205 ± 4*
CITALOPRAM					
0.1	6	102 ± 2	104 ± 2	242 ± 4	235 ± 2
0.3	6	106 ± 3	98 ± 1*	235 ± 3	225 ± 2*
1	6	104 ± 2	87 ± 1*	248 ± 4	226 ± 2*

TABLE 8. Maximal changes induced in MABP and HR by fluoxetine or citalopram administration to 6 awake rabbits. Mean maximal values are given ± SEM. *n* = number of animals. *$p < 0.05$ *vs* baseline values.

Discussion

This was the first published study of the effects of human therapeutic doses of two SSRIs, fluoxetine and citalopram, on the normal and ischemic cCBF in conscious rabbits. The rabbit seems to be an ideal animal for the study of cCBF changes because of the very similar cerebral vasculature and hemodynamics to those of primates[37].

In our experiments, neither fluoxetine nor citalopram exerted a significant influence on the normal cCBF. Fluoxetine administration does not have a discernible effect on mood in normal subjects[17]. Similarly, the CBF was only minimally affected by fluoxetine treatment: no change (from the baseline) in global or regional CBF was observed in healthy subjects treated with fluoxetine for 6 weeks[6]. These findings suggest that mood-enhancing and other psychoactive effects are not general properties of the SSRIs, but are manifested in the context

of target symptoms. Indeed, some data suggest that fluoxetine and also citalopram may be effective in the treatment of PSD[79].

The effects of fluoxetine or citalopram on the cCBF in the present work differed under ischemic conditions. Whereas the fluoxetine doses applied did not exhibit a significant effect on the cCBF, there was only a very small tendency to improve it; citalopram dose-dependently increased the cCBF in the animals subjected to unilateral carotid occlusion. It is of interest that there have been reports of reductions in the CBF and metabolism to support a hypothesis associating depression with reduced brain perfusion[30]. Sackeim et al.[63] described a marked reduction in global cCBF in the depressed group, and an abnormality in the topographic distribution of the blood flow. Consequently, the cCBF-improving effect of citalopram might be considered a factor contributing to the antidepressant feature of this drug.

Multiple and complex interactions exist between the cerebral circulation and the potent vasoactive (and neurotransmitter) agent SER, the endogenous substance accumulating in the presence of citalopram or fluoxetine. The cerebral blood vessels are richly innervated by SER-ergic nerve fibers. It has been demonstrated that the brain endothelium (of capillaries and/or larger vessels) may serve as a specific target for SER. The local effects of SER on the blood vessels of the tissues where it is formed and released reveal a duality of its vascular effects, causing either constriction or dilatation, depending on the vascular bed studied and the experimental conditions[80]. Although SER results in the dilatation of small cerebral arterioles, it has a potent constrictive effect on large cerebral arteries, such as the basilar artery. It is also well known that SER plays an important role in several pathological conditions, including not only depression, but also migraine, cerebral vasospasm and cerebral ischemia[27]. Cerebral small vessel disease has been postulated as a potential cause of depression. SER may be involved in the maintenance of the CBF within normal limits (autoregulation to perfusion pressure or blood gas changes)[33].

As concerns ischemic stroke, it is pertinent that SER is released from the platelets to promote vasoconstriction and platelet aggregation in response to vascular tears[7]. During treatment with SSRIs, the intraplatelet SER content is reduced as a result of inhibition of the SER reuptake mechanism. Conversely, it is conceivable that SSRIs might reduce the risk of thrombus formation, although the effect of SSRI exposure on the risk of ischemic stroke has not been studied previously. To clarify the role of SER in cerebral ischemia, Nakata et al.[45]

examined the effects of two SSRIs, citalopram and clomipramine, on ischemic neuronal damage in the gerbil. The striking increases in the EAA levels during ischemia were significantly inhibited by pretreatment with citalopram, suggesting that the protective effect is mediated through prevention of the accumulation of extracellular EAAs during and after ischemia. Wegener et al.[83] proposed that SER-ergic antidepressants at the dosages used clinically do not directly affect NOS, but the findings may reflect a secondary action of antidepressants on the Glu (NMDA) receptor following their primary inhibitory action at the SER transporter.

Previous studies have shown that fluoxetine dilates isolated rat small cerebral arteries independently of the endothelium, probably by interfering with Ca^{2+} entry[78]. This *in vitro* dilatatory effect of fluoxetine could therefore result in an increase in the CBF *in vivo*. Fluoxetine exhibits cardiodepressant and vasodilatory effects in isolated heart preparations and vessels of rabbits[49]. The inhibition of cardiac Ca^{2+} and Na^{+} channels may explain most of the cardiac side-effects of the drug. The HR-decreasing effect of fluoxetine and citalopram observed in our experiments may also be relevant to this action. However, it is unlikely that the mild alterations in the peripheral circulatory parameters detected in our study could efficiently influence the cCBF. The results of Terstappen et al.[77] indicate a direct molecular interaction of fluoxetine with human small-conductance Ca^{2+}-activated potassium channels, suggesting the possibility that blockade of these channels by fluoxetine may be important in its therapeutic action or undesired side-effects.

In conclusion, we found that citalopram and, to a very slight extent, fluoxetine increased the cCBF in rabbits in which the cCBF had been impaired by unilateral carotid occlusion. Cerebral ischemia is associated with marked rises in the levels of EAAs such as Glu, and the overstimulation of Glu receptors leads to an abnormal elevation in $[Ca^{2+}]_i$ in the neurons, an event thought to be important in ischemic neuronal death. The additional antiplatelet, Ca^{2+}-channel-inhibitory, NMDA receptor antagonistic properties of citalopram and fluoxetine may play a role in the beneficial effect of these drugs in the animals with a reduced cCBF induced by unilateral carotid occlusion in our experiment. The exact mechanism by which citalopram improves the cCBF needs further investigations. The SSRIs, and in particular citalopram, may furnish advantages in the treatment of PSD and vascular depression.

SUMMARY AND CONCLUSIONS

L-KYN (300 mg/kg) + PROB (200 mg/kg) i.p. pretreatment caused significant neuroprotection in a global ischemic rat model, while its effect as post-treatment was moderate. L-KYN (0.1, 1 and 3 mg/kg i.v.) led to an increase in cCBF in both the control rabbits and the animals with chronic cerebral ischemia induced by chronic unilateral carotid artery occlusion. While the hippocampal neuron rescue effect of L-KYN could be due to its Glu antagonistic property, the mechanism of its cCBF-enhancing effect remains to be elucidated. Involvement of the cholinergic pathways of the cranial parasympathetic nervous system could be assumed, in which vasodilation is related to the release of NO, as atropine, an anticholinergic drug, or L-NAME, a NOS inhibitory drug, attenuated the cCBF-increasing effect. In any case, this effect could have an additional beneficial impact on the disease outcome under ischemic conditions in the brain.

In another study, we investigated whether the SSRI drugs fluoxetine and citalopram (0.1, 0.3 and 1 mg/kg) influence the cCBF in doses corresponding to that used in clinical settings. We found that only citalopram had a significant effect on the cCBF: it improved the unilateral carotid occlusion-induced impaired cCBF response. Besides the possibility of the blocking of Ca^{2+} and/or Na^{+} channels on cerebral arteries by SSRI drugs, several authors have proposed that these drugs can exert a secondary (inhibitory) action on the Glu - NMDA receptors in addition to their primary inhibitory action at the SER transporter. Taken together, the cation channel-inhibitory or NMDA receptor antagonistic properties of citalopram may have played a role in the beneficial effect of the drug in the animals with an impaired cCBF induced by unilateral carotid occlusion in our experiments. Both fluoxetine and citalopram slightly decreased the HR, whereas higher doses of citalopram reduced the MABP under either normal or unilateral carotid occlusion-induced ischemic conditions. However, it is not likely that the mild alterations in the peripheral circulatory parameters could efficiently influence the cCBF. On the basis of our findings, citalopram may have advantages in the treatment of PSD and other types of vascular depression, but further studies of the exact mechanism by which the drug improves the cCBF are clearly necessary.

Details of our results can be itemized as follows:

1. In our 4VO rat ischemia model, there was severe neuronal damage in the CA1 area of the hippocampus in both hemispheres 10 days after the intervention, as proved by FJ-B staining

and NeuN immunohistochemistry. The CA3 area and the dentate gyrus remained intact. KYN (300 mg/kg, i.p.) administered together with PROB (200 mg/kg, i.p.) considerably decreased the number of injured neurons in the CA1 region. However, the decrease in the number of injured neurons was highly significant only in the pretreated group (to 52% relative to the damaged cells induced by 4VO without KYN+PROB treatment). The animals in the post-treated group also exhibited a tendency to a reduction in the number of injured neurons, but this change was not significant. The NeuN immunohistochemistry results were in accord with those observed with FJ-B staining: in the 4VO model, post-treatment with KYN had hardly any effect, while KYN pretreatment proved to be highly neuroprotective: the number of intact cells was comparable to the control level.

2. Our *in vitro* electrophysiological studies indicated that in 4VO rats, there was no significant difference between the IO curves in the three groups, implying that the basal functions of the registered pyramidal cells and synapses were not affected by complete ischemia. In the majority of the 4VO animals, no LTP was observed. The administration of KYN and PROB protected the brain slices from the 4VO-induced LTP impairment. KYN restored the fEPSP slopes to the control level, and these parameters were stable until 60 min after HFS.

3. Administration of KYN (300 mg/kg) + PROB (200 mg/kg) to 4VO rats considerably increased both the KYN and KYNA concentrations in the plasma and brain, and also altered their proportions within the brain compartments studied. The KYN concentration increased 37-fold in the plasma and approximately 70-fold in the hippocampus and cortex, while the KYNA concentration was elevated roughly 300-fold in the plasma and 50-fold in the hippocampus and cortex.

4. Chronic unilateral carotid occlusion caused a significant reduction in cCBF in awake rabbits (from 117±15 to 57±11 (“1”), from 90±12 to 52±8 (“2”) and from 113±10 to 58±11 (“3”) ml/min/100 g tissue). L-KYN administered intravenously in single doses of 0.3, 1 and 3 mg/kg resulted in a significant increase in the cCBF in the control rabbits, and also in the animals with unilateral carotid occlusion. The effect was particularly obvious after the administration of 1 mg/kg L-KYN.

5. Pretreatment with atropine (1 mg/kg) or L-NAME (40 mg/kg) prevented the cCBF-increasing effect of 1 mg/kg L-KYN in control rabbits and also in those with chronic unilateral carotid occlusion.

6. Both SSRI drugs fluoxetine and citalopram (in individual doses of 0.1, 0.3 and 1.0 mg/kg, which correspond to the therapeutic human doses) caused only minor, insignificant changes in the normal cCBF in rabbits.
7. Citalopram (0.1, 0.3 or 1 mg/kg in infusion) improved the unilateral carotid occlusion-induced impaired cCBF response as compared with the basal occluded values. The effect was dose-dependent; the most pronounced change was seen in response to 1 mg/kg citalopram, *i.e.* the highest applied dose of the drug.
8. Both fluoxetine and citalopram decreased the HR as compared with the baseline value.
9. Significant reductions in MABP were observed in the groups treated with 0.3 or 1 mg/kg citalopram under either normal or unilateral carotid occlusion-induced ischemic conditions.

KYNA-like agents – as potential antiglutamatergic drugs for the future

Glu is a predominant excitatory transmitter in the central nervous system. Glu-ergic neurotransmission plays an important role in many physiological processes, such as learning, development of the nervous system, moving, sensation and mood control. Under particular circumstances, its extreme release may cause irreversible damage to the neurons through their overexcitation. This is termed excitotoxicity and is involved in the pathogenesis of many diseases which are apparently different in nature (stroke, neurodegenerative and mood disorders, *etc.*). In these cases, a therapeutic elevation of the brain KYNA content may hold promise to diminish the consequences of excitotoxicity. On the other hand, it must be mentioned that in diseases with cognitive alterations, such as Alzheimer's dementia and schizophrenia, elevated brain KYNA levels have been reported which could contribute to the cognitive defects by interfering with the NMDA receptor function.

Our experiments demonstrated that KYN treatment abates neuronal cell loss in a rat global ischemia model, in the pathogenesis of which excitotoxicity is assumed. In addition, low doses of L-KYN enhance the cCBF in rabbits under both normal and ischemic conditions of the brain. Citalopram, an SSRI drug, increased the cCBF only in rabbits with unilateral carotid occlusion. Its precise mechanism is still unclear, but an NMDA antagonistic effect of the drug may be involved. Our findings strengthen the data which indicate that KYNA or one of its derivatives with better bioavailability may be fruitful in the treatment of certain acute or chronic neurological diseases involving Glu receptor hyperactivity in their pathomechanism.

POTENTIAL CLINICAL RELEVANCE OF THE RESULTS

The pathological overactivation of Glu receptors plays a role in the pathogenesis of numerous neurological and psychiatric diseases. Abatement of the excitotoxic process can retard the disease course and diminish its damaging consequences.

In our experiments, L-KYN, an NMDA receptor antagonist that is produced within the body, proved to be neuroprotective in a transient global ischemic animal model. Although NMDA receptor antagonists failed in most acute stroke trials, as they suspend even the physiological NMDA receptor function and thereby cause severe side-effects, smaller doses of L-KYN administered for a longer period of time may theoretically halt the neurodegenerative process, and (as this is an endogenous substance) may lack toxic side-effects.

In the event of therapeutic administration, the additional CBF-increasing effect of L-KYN under chronic cerebral ischemic conditions may provide further benefit.

Hyperactivity of the NMDA receptors in the cortico-hippocampal pathways in depression may be presumed, a precise understanding of which may hold further therapeutic perspectives. Citalopram, an SSRI antidepressant drug, dose-dependently increased the CBF in animals, a feature in favor of its use in PSD.

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